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Fine structure of the middle ear epithelium in the chicken (*Gallus gallus*)

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ABSTRACT

The epithelium lining the tympanic cavity of the chicken possesses distinct morphological characteristics. Its ultrastructure was studied using 2 preparative techniques. (1) After fixation in Karnovsky's solution, postfixation in osmium tetroxide and embedding in Epon, the epithelium was observed to contain 2 kinds of cell: secretory and basal. The secretory cells (which we refer to as mixed granulated cells) showed numerous secretory vesicles that varied in appearance, some containing paracrystalline formations. The basal cells, located close to the basement membrane, showed no evidence of secretory activity. (2) Other specimens were immersed in Karnovsky fixative and subsequently in a mixture of glutaraldehyde and tannic acid. They were then osmicated and embedded in polar Epon mix. With this method, the epithelium was seen to be covered by electron-dense material made up of thin intertwined tubules. In addition, the secretory cells contained vesicles with concentrically arranged lamellae; such vesicles resembled the multilamellar bodies of mammalian type II pneumocytes. The hypothesis is advanced that tubules and lamellar vesicles are related to the presence of surfactant substances.

INTRODUCTION

The morphological and functional characteristics of the epithelium of the middle ear of mammals have been the subject of a number of investigations. It has been established that, in the eustachian tube, the epithelium is pseudostratified and respiratory in nature and is therefore characterised by both basal cells and by goblet and ciliated cells (Hentzer, 1970; Lim, 1974, 1976; Albiin, 1984). This epithelium also extends to the interior of the tympanic cavity, partly lining the mucosa in ways that differ from species to species (Sade, 1966; Albiin et al. 1986). On the inner face of the tympanic membrane, on the other hand, the epithelium is simple squamous and is made up of cells of great cytological and, presumably, functional simplicity (Johnson et al. 1968; Lim, 1970). The remaining parts of the middle ear are lined by a pseudostratified epithelium which is different from a respiratory epithelium and which possesses special cytological characteristics (Hussl & Lim, 1969; Lim & Hussl, 1969; Lim & Shimada, 1971; Lim, 1974; Lim et al. 1976). It has been reported to contain 'basal cells' which, located close to the basement membrane, are situated at a distance from the surface of the epithelium. They are commonly considered to be replacement cells. The other cells, without motile cilia, extend as far as the lumen of the tympanic cavity and show clear signs of secretory activity; these have been considered to be responsible for the production not only of mucus but also of lysozyme (Lim et al. 1976) and surface tension-lowering substances (surfactants) (Birken & Brookler, 1972; Rapport et al. 1975; Hills, 1984; Tsuruhara et al. 1989). In the classification of these secretory cells, authors have, in general, turned their attention to the ultrastructural appearance of the secretory vesicles and have identified 3 types of cell: light, dark and intermediate. The first are considered to produce mucus and lysozyme and are characterised by light PAS-positive secretory vesicles, whereas the dark cells produce one or more substances of a proteinaceous nature and possess electron-dense and PAS-negative secretory vesicles. Finally, the characteristics of the intermediate cells are somewhere between those of the 2 previous types, insofar as they contain both light and dark vesicles. The structure of the epithelium of the middle ear of birds, unlike that of mammals, is not known exactly; the epithelium in

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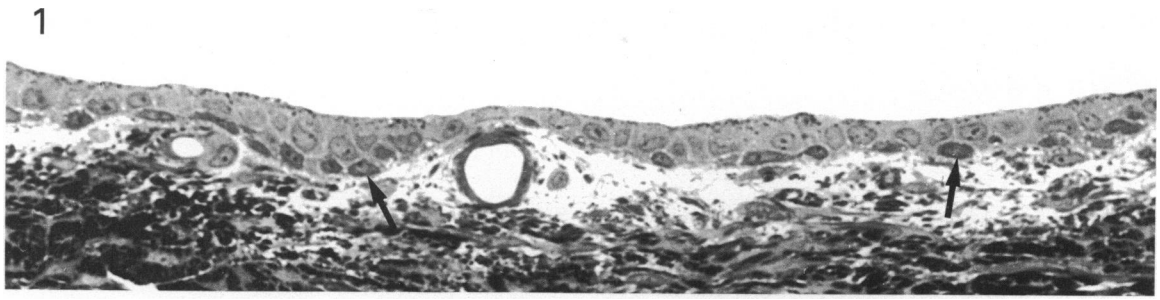


Fig. 1. Epithelium of tympanic cavity of the chicken processed by method A. The epithelium lies on a thin layer of loose connective tissue, below which is a dark-coloured layer of dense connective tissue. The epithelium is cuboidal or flattened in shape. The arrows point to the dark basal cells. ×870.

the eustachian tube is probably of a respiratory nature, but it is not known if or how this extends into the tympanic cavity; such problems will be examined, if necessary, in future research. The aim of the present paper is to ascertain if the tympanic cavity contains a secretory epithelium that differs from the respiratory epithelium and, if so, to describe its ultrastructural characteristics.

MATERIAL AND METHODS

In the present study we used adult Hubbard chickens of both sexes. After the animals had been anaesthetised with pentobarbital, fragments of mucosa were taken from the various walls of the tympanic cavity, excluding the tympanic membrane. The fragments were then fixed and embedded using 2 different methods.

Method A

The fragments of mucosa were fixed by means of Karnovsky fixative (Karnovsky, 1965) for 1 h. After a quick washing in the buffer, the specimens were postfixed in osmium tetroxide (1.3%) in cacodylate buffer, pH 7.4, for 1 h. Finally, the specimens were dehydrated in alcohol, immersed in propylene oxide for 1/2 h and embedded in Epon 812.

Method B

Using the method described by Stratton (1976), the specimens were fixed for 30 min using Karnovsky fixative and subsequently immersed in a mixture of glutaraldehyde (1%) and tannic acid (1%) in 0.1 M cacodylate buffer, pH 7.3 for 1 h. The specimens were osmicated and finally dehydrated with Epon 812 and embedded in polar Epon mix. In greater detail, for embedding they were immersed in 90% Epon 812 for 2 h, 100% Epon 812 overnight, 1:1 Epon 812: polar Epon mix, 2 h, and 100% polar Epon mix, 3 h. The specimens were then encapsulated and polymerised at 60° for 48 h. The composition of the polar Epon mix was Epon 812, 50 ml; NMA, 42 ml; DMP 30, 1.2 ml. This method of fixing and embedding has been used for research on the alveolar surfactants of mammals (Stratton, 1975, 1976). As is well known, this is a complex mixture of lipids, proteins and carbohydrates in a relationship which varies from species to species. The lipids form 85–90% of the surfactant and are made up mainly of phospholipids (~ 90%); phosphatidylcholine is the predominant phospholipid and it alone accounts for 70–80% of the lipid fraction of the surfactant. At present, phosphatidylcholine is considered to be the main surface-active component of the surfactant. It has been ascertained that tannic acid reacts with the phospholipids to form compounds

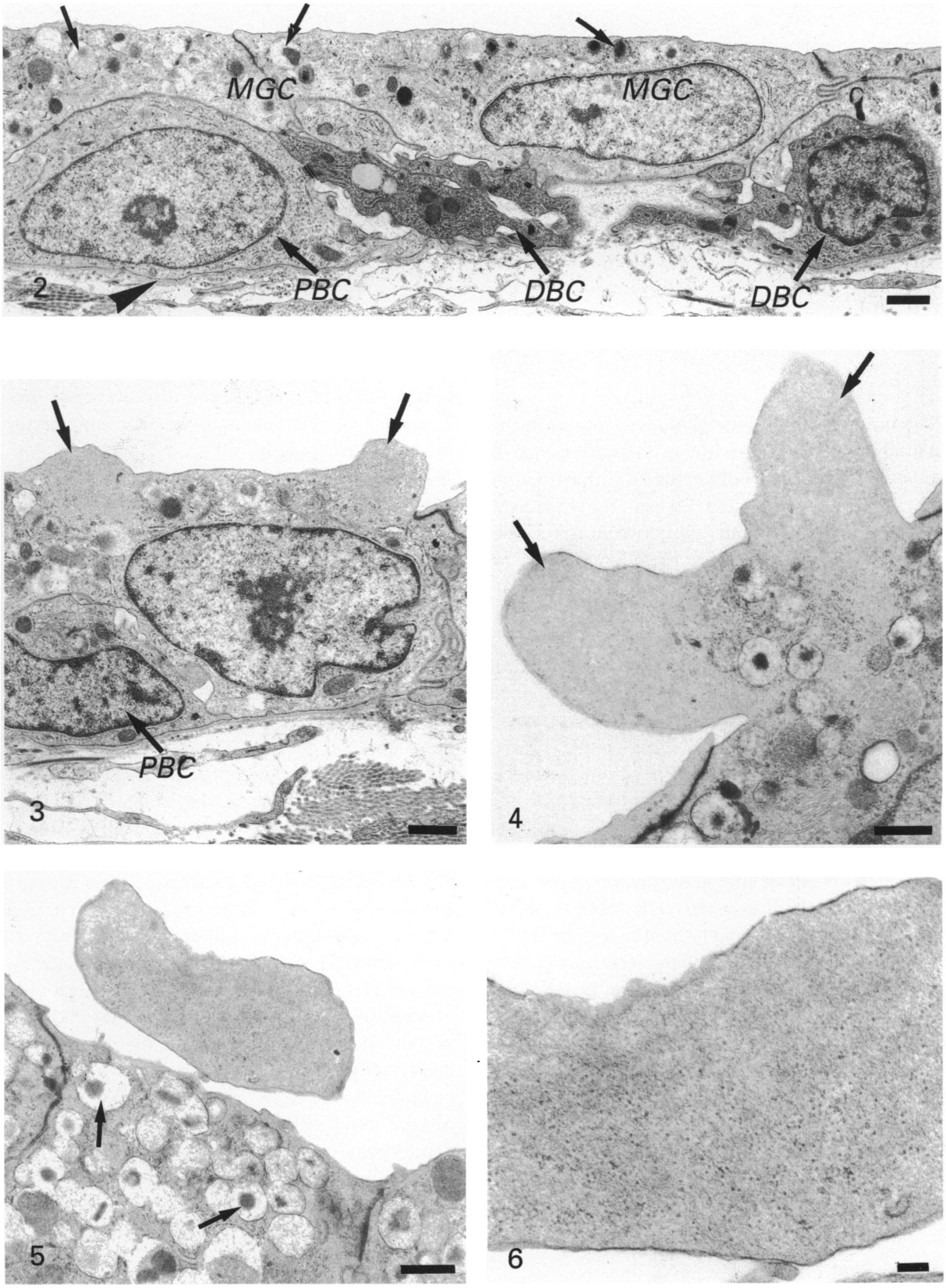
Fig. 2. Low magnification electron micrograph of the epithelium of the tympanic cavity of the chicken processed by method A. The epithelium is flattened and has a few basal cells, located close to the basal lamina (arrowhead). Two dark basal cells (*DBC*) and 1 pale basal cell (*PBC*) can be observed. The other cells, mixed granulated cells (*MGC*), extend as far as the surface of the epithelium. Within them are a number of vesicles with a content of variable density (arrows). Bar, 1 μm.

Fig. 3. Epithelium of tympanic cavity (method A) showing an MGC on whose apical surface are irregular protrusions (arrows) made up of homogeneous light-coloured material. *PBC*, pale basal cell. Bar, 1 μm.

Fig. 4. Epithelium of tympanic cavity (method A) showing the apical part of an MGC with 2 protrusions similar to those in Figure 3 (arrows) and which appears to be pedunculated. Bar, 0.5 μm.

Fig. 5. Apical part of an MGC (method A). The cell is characterised by numerous secretory vesicles (arrows). A protrusion juts out from the epithelium, and has a content similar to that of the protrusions in Figures 3 and 4. Bar, 0.5 μm.

Fig. 6. Higher magnification view of the protrusion seen in Figure 5. It contains finely granular material. Bar, 0.1 μm.



Figs 2–6. For legend see opposite.

which are subsequently stabilised by treatment with osmic acid. In their turn, dehydration with Epon 812 and embedding in polar Epon mix prevent solubilisation of these compounds by alcohol and by propylene oxide. It is just possible that tannic acid may also interact with the protein and carbohydrate components of the surfactant, helping to bring about fixation. Ultrathin sections of the epithelium were cut on a Porter Blum Ultrotome, double stained with uranyl acetate and lead citrate and observed through a Siemens Elmiskop I. Semithin sections ~ 1 μm in thickness were cut on the same Porter Blum Ultrotome and stained with toluidine blue dissolved in 1 % borax.

RESULTS

No significant regional differences were found in the structure of the epithelium in the tympanic cavity of the chicken. It was generally cuboidal or flattened in shape. It had no cilia and lay on the basement membrane (Figs 1–3). Some parts of the epithelium appeared to be just 1 cell thick. In other regions the epithelium contained squamous cells located close to the basement membrane and separated from the lumen of the tympanic cavity by the other types of cell of the epithelium. The basal cells could be divided into pale and dark types. Indeed, some cells contained dark cytoplasm (DBC, Fig. 2), whereas others were pale, as were the more superficial ones (PBC, Figs 2, 3). These basal cells did not contain secretory vesicles. The other cells of the epithelium protruded into the lumen of the tympanic cavity and were generally squamous or cuboidal in shape. They possessed a large variety of vesicles and, in the present paper, they will be termed *mixed granular cells* (MGC). When these MGC were fixed using technique A (Figs 2–13), the following ultrastructural characteristics were observed. On the apical surface, which is generally smooth and free from microvilli, there were irregular protrusions in a few cells (Figs 3–6) and these contained light, finely granular material (Fig. 6). Sometimes the apical part of the cells containing this finely granular material appeared to be pedunculated (Fig. 4) and to jut out from the surface of the epithelium (Fig. 5). The vesicles were rounded or oval in shape and 0.5–2 μm in diameter. In a few instances they contained material of homogeneous electron density (Fig. 7); more frequently, their outer area was light and their centre darker (Fig. 8). A typical feature of these vesicles is shown in Figures 9 and 10 and consisted of the presence of paracrystalline formations made up of thin lamellae arranged in parallel array. The lamellae were located in the central dark part of the vesicles and filled it completely (Fig. 9) or only partly (Fig. 10).

In order to define the periodic fine structure of these paracrystalline formations, we measured the centre–to–centre spacing of the lamellae. This distance remained approximately constant in any one vesicle, but could vary slightly between one vesicle and another. Indeed, in the vesicle in Figure 9 this distance is ~ 30 nm, but in the paracrystalline formation in Figure 10 it is ~ 23 nm. Closely packed tubules set in a regular geometrical array were found in other vesicles (Fig. 11); each tubule was surrounded by 6 identical tubules arranged hexagonally. Multivesicular bodies were frequently found in the MGC; within them, in several instances, we observed a number of small vesicles and also accumulations of granular material similar to that encountered in the secretory vesicles (Fig. 12).

In specimens embedded by technique B (Figs 14–19), we observed that the use of tannic acid sometimes made it possible to identify new ultrastructural features in the epithelium. The epithelium appeared to be covered by dark material of varying thickness (Fig. 14) which, at high resolution, proved to be made up of numerous thin tubules. These tubules were in contact with the surface of the epithelium and were irregularly intertwined (Fig. 15). The secretory vesicles sometimes contained electron-dense material, morphologically similar to that found on the surface of the epithelium (Fig. 15). At other times, the appearance of the secretory vesicles was similar to that found using technique A (Fig. 16). Moreover, examination of the multivesicular bodies and the secretory vesicles suggested there is a further correlation between these organelles, even though certain morphological aspects were different from those observed after treatment with technique A. In the multivesicular bodies we found not only a number of small vesicles but also irregular inclusions made up of some membranes and granular material of variable density (Fig. 17); similar structures were also encountered in the secretory vesicles (Fig. 18). Lastly, in certain secretory vesicles we observed an electron-dense body made up of concentrically arranged lamellae; these lamellae were separated from the membrane which surrounds the vesicle by a clear space containing a few of the tubular formations (Fig. 19).

DISCUSSION

This study has demonstrated that an epithelium with distinct morphological characteristics is present in the

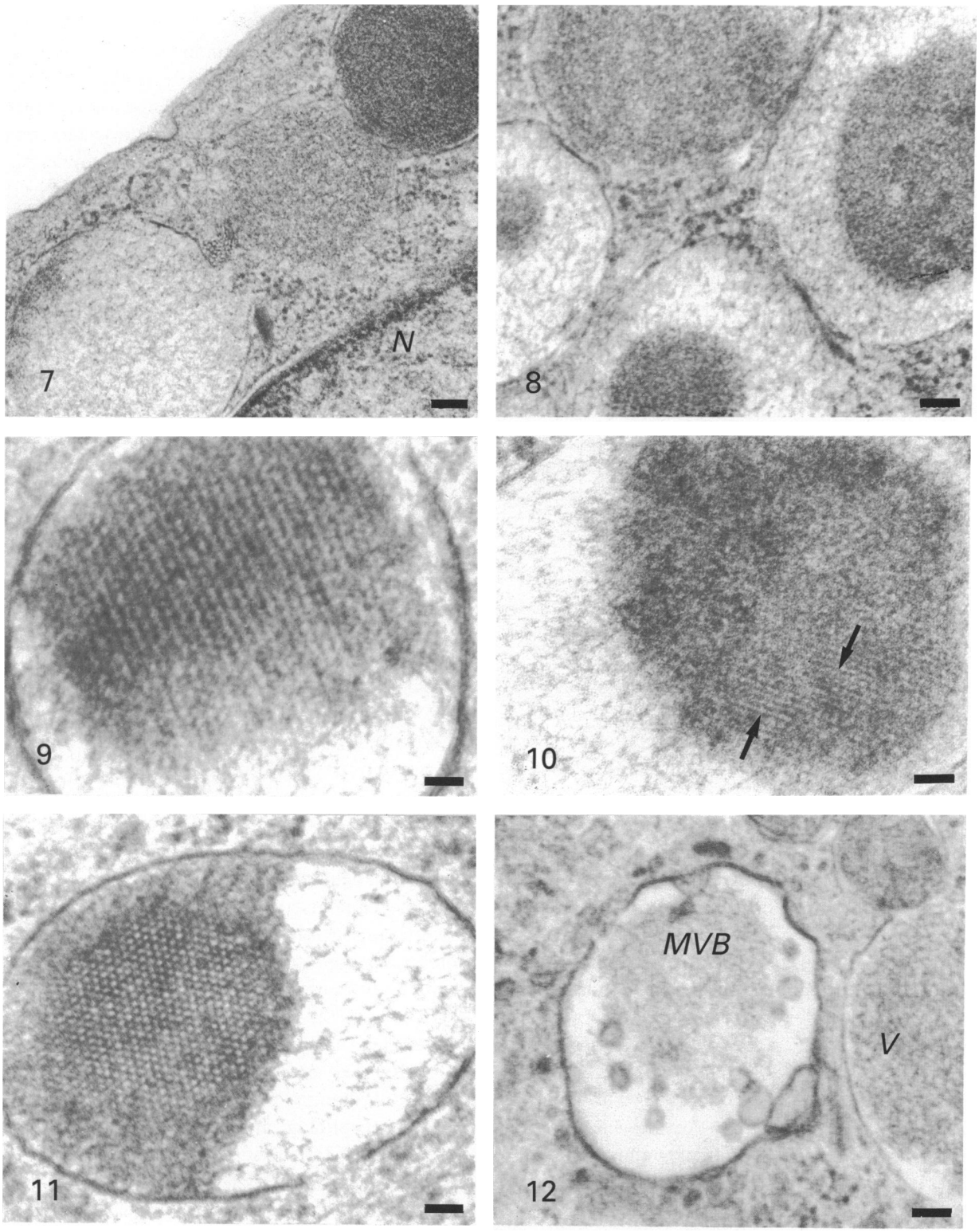


Fig. 7. Method A. Three vesicles contain finely granular material of variable electron density. Numerous free ribosomes are also present; *N*, nucleus. Bar, 0.1 μm.

Fig. 8. Method A. Unlike those in Figure 7, these vesicles are characterised by a dark central part of granular appearance and by a light outer area the contents of which are granulofilamentous. Bar, 0.1 μm.

Fig. 9. Secretory vesicle, method A. The central part contains thin lamellae arranged in parallel formation. Bar, 0.1 μm.

Fig. 10. Secretory vesicle, method A. The lamellae (arrows) occupy only a part of the central area, the remainder of which is made up of granular material. Bar, 0.1 μm.

Fig. 11. Secretory vesicle, method A, containing dense material situated off-centre and made up mostly of thin closely packed tubules, the diameter of which is ~ 24 nm. Bar, 0.1 μm.

Fig. 12. Method A. A multivesicular body (*MVB*) contains not only a few small vesicles but also granular material similar to that found in the secretory vesicles. *V*, secretory vesicle. Bar, 0.1 μm.

tympanic cavity of the chicken. It differs both from typical respiratory epithelium because of the absence of ciliated and goblet cells, and from the secretory epithelium found in the tympanic cavity of mammals. We found that the so-called dark granulated cells are absent in chicken; these cells, on several occasions, have been reported to exist in the tympanic cavity of mammals; they have PAS-negative electron-dense secretory vesicles and are similar to the serum-secreting cells in the glands of the eustachian tube (Hussl & Lim, 1969; Lim & Shimada, 1971; Lim, 1974, 1976).

Although the MGC are reminiscent of the intermediary cells of mammals, the paracrystalline formations described in this study are a morphological characteristic which differentiates the 2 types of cells. Our observation that there seem to be 2 types of paracrystalline formation in the MGC does not necessarily mean that these are distinct structures. Indeed it is likely that what appears to be a lamellar configuration of some of the vesicles (Figs 9, 10) is an oblique view of the hexagonal formations seen in others (Fig. 11) and that the secretory vesicles are of the same type. The appearance and the size of the tubules and lamellae support this interpretation.

Paracrystalline formations, similar to those that we have seen in the MGC, are found in the endocrine pancreas (Grossner, 1967; Lange, 1973, 1974). Indeed, in many vertebrate species the B cell secretory vesicles of the islets of Langerhans possess a periodic structure in their electron-dense core. This may be either lamellar in appearance or tubular, the tubules being arranged hexagonally. These structures are similar to those in Figures 9–11. However, the periodicity of the lamellar structures differs. This has been examined in the B cells of several vertebrates in a review by Lange (1973). Adopting the same criteria of measurement that we used for the MGC, Lange reported periodicity values ranging from 3 to 11 nm; they are therefore lower than those in the MGC of the chicken. It is generally believed that the paracrystalline structures of the B cells are formed by insulin or an insulin-protein compound (Lange, 1973). Considering the similarity of the paracrystalline formations present in the 2 cell types, we can reasonably advance the hypothesis that, in the MGC also, these structures are formed by a proteinaceous substance.

After treatment with tannic acid (technique B), we noted other interesting features of the MGC, so far not found in the epithelium of the tympanic cavity of mammals. Some of these features may be related to the secretion of surfactants. Birken & Brookler (1972) identified surfactants in secretions taken from the middle ear of dogs; these findings were subsequently confirmed by Rapport et al. (1975), Hills (1984) and Tsuruhara et al. (1989). Following the work of Birken & Brooker, an attempt was made to throw light on the cytological phenomena related to the production of the surfactant. Special attention was paid to vesicles which resemble the multilamellar bodies. As is well known, the latter are vesicles which are located in type II pneumocytes of mammals and which contain the alveolar surfactant (Sorokin, 1967; Chevalier & Collet, 1972). Electron-dense material characterised by thin closely-packed lamellae, arranged concentrically, is present within them. Vesicles of this kind were encountered by Lim & Shimada (1971) in the intermediate cells of the tympanic mucosa. These findings were sporadic and the material involved was, at least in part, pathological, whereas in the MGC of chicken vesicles such as the one shown in Figure 19, with a structure similar to that of multilamellar bodies, are frequently found. However, they are only readily identified when tannic acid is used during fixation. This may be related to the presence of phospholipids with which, as stated above, tannic acid forms compounds, thereby making their fixation easier (Kalina & Pease, 1977*a*, *b*). Another interesting feature of the MGC is that the content of the secretory vesicles and that of the multivesicular bodies is often morphologically similar. In this connection, observations on type II pneumocytes of mammals have shown that coalescence frequently takes place between the multivesicular bodies and the secretory vesicles so that the contents of these organelles can mix. At present, this cytological process is thought to constitute an important phase in the synthesis and recycling of the surfactant (Sorokin, 1967; Chevalier & Collet, 1972; Post & Van Golde, 1988). Our observations lead us to think that such a phenomenon may also take place in the MGC. The very large number of tubules that cover the epithelium constitute a novel morphological feature, but insufficient data are available to allow us to give a definitive interpretation of these structures. On the other hand, their being observable is clearly linked to the use of tannic acid, since they can be seen only when the specimens are prepared using technique B. The possibility that the tubules are made up of phospholipids and that they are related to the presence of a surfactant therefore cannot be excluded. Our hypothesis is supported by a recent study by Schrijvers et al. (1989) who found that tannic acid reduces the extraction of phosphatidylcholine during the process of fixation. Moreover, the addition of tannic acid in vitro to vesicles made up of phosphatidylcholine

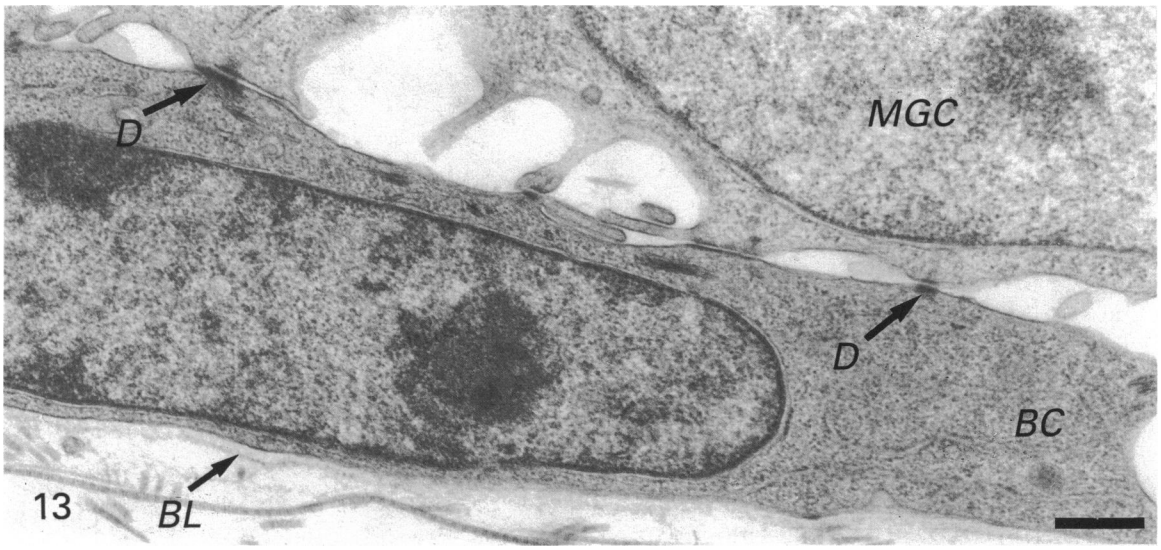


Fig. 13. Basal part of the epithelium, method A. A flattened basal cell (*BC*) lies on a basal lamina (*BL*). Secretory vesicles are not present in the cytoplasm. The basal cell is linked to a mixed granulated cell (*MGC*) by a few desmosomes (*D*). Irregular microvilli protrude into the space between the 2 cells. Bar, 0.5 μm.

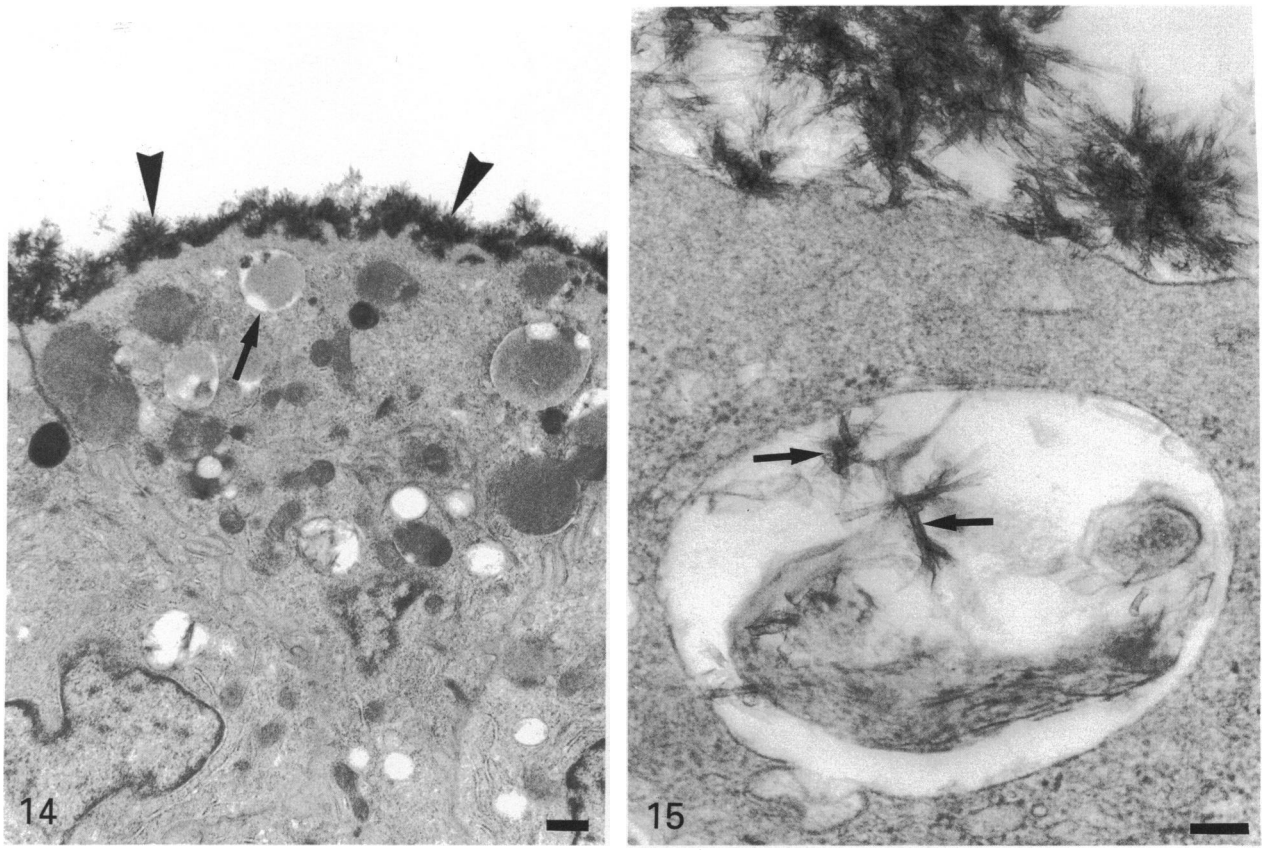


Fig. 14. Method B. Dark-fringed material of variable thickness (arrowheads) can be seen on the surface of the epithelium. Numerous secretory vesicles are present in the MGC. The arrow indicates a secretory vesicle shown at higher magnification in Figure 18. Bar, 0.5 μm.

Fig. 15. Method B. At high magnification the material covering the epithelium is seen to be made up of thin irregularly intertwined tubules. A vesicle is present in the cytoplasm and contains tubules that are identical to those seen on the surface of the epithelium (arrows). Bar, 0.1 μm.

causes the formation of multilamellar structures which would otherwise not be visible. Our observations show that tubules are to be found within the vesicles of the MGC (Figs 15, 18). These structures therefore do not consist of a nonspecific precipitate at the surface of the epithelium but are probably formed by

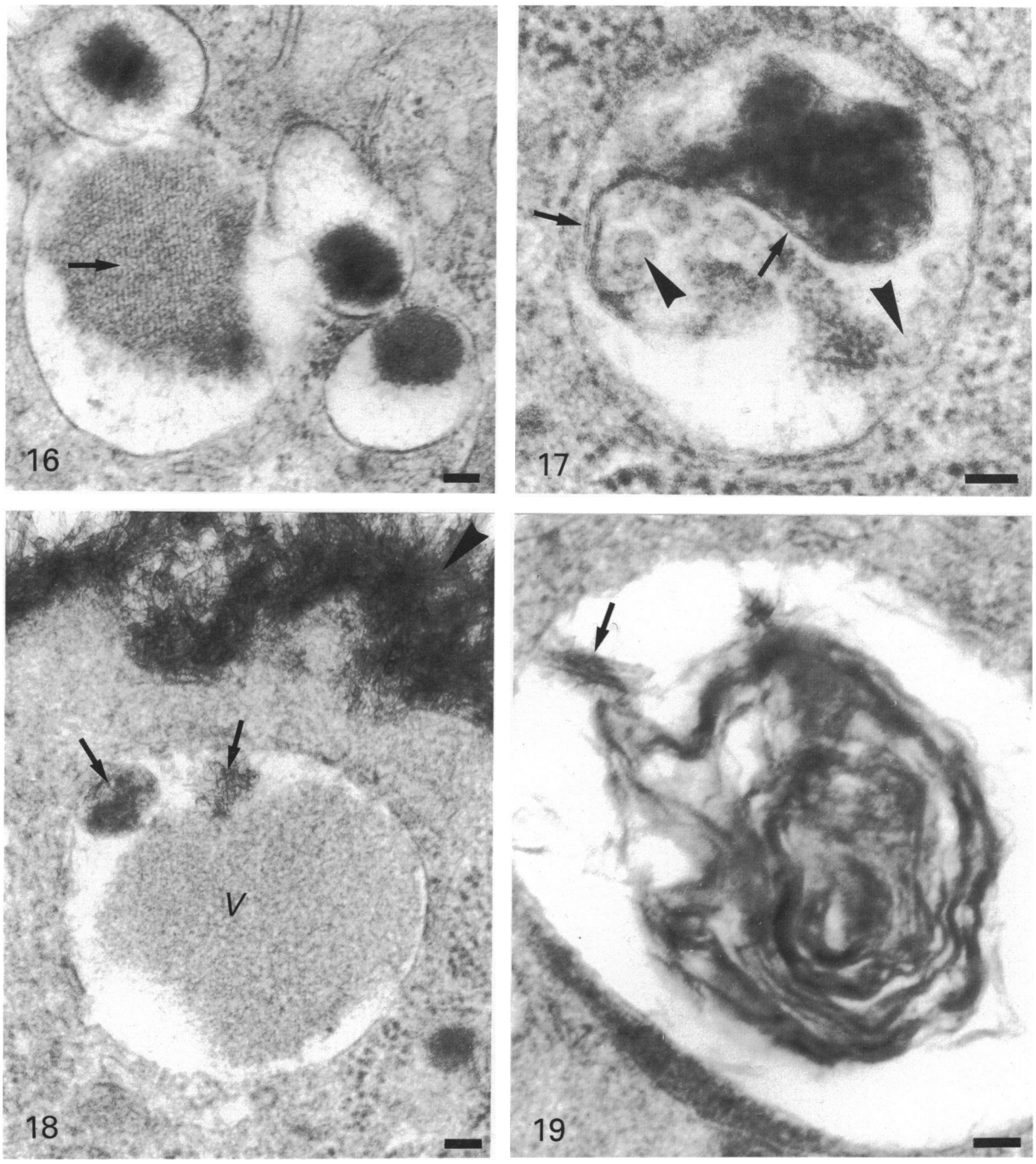


Fig. 16. Method B. This figure shows a group of secretory vesicles similar in appearance to those processed by technique A. The arrow points to a paracrystalline formation. Bar, 0.1 μm.

Fig. 17. Method B. Multivesicular body containing a few vesicles (arrowheads), granular material of variable density and a number of membranes (arrows). Bar, 0.1 μm.

Fig. 18. Detail of Figure 14. The secretory vesicle (*V*) contains a light granular substance and 2 electron-dense formations (arrows); the one on the left is similar to the dense content of the multivesicular body in Figure 17; that on the right has the same appearance as the electron-dense material covering the epithelium (arrowhead). Bar, 0.1 μm.

Fig. 19. Method B. The central part of the vesicle shown in this figure is characterised by concentrically-arranged electron-dense lamellae, whereas the outer part consists of a clear space containing tubules similar to those covering the epithelium (arrow). The tubules appear to jut out from the lamellar material. Bar, 0.1 μm.

material, possibly phospholipids, secreted by the MGC. With regard to the protrusions on the apical part of the MGC, our findings suggest that the material produced by the cells may be released into the tympanic cavity by a process of progressive detachment. Secretion of an apocrine nature by the

MGC would thus seem to occur. This hypothesis is supported by the observations of Lim & Shimada (1971) who reported appearances similar to ours. They reached the same conclusions from a functional approach in their study on the ultrastructural features of the mucosa of the tympanic cavity in man.

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